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Xanthene-dye-labelled phosphatidylethanolamines as probes of interfacial pH

Studies in phospholipid vesicles

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We have been developing the use of plasma-membrane-bound fluorescent probes to measure the pH values at the surfaces of living chondrocytes. For this purpose, three lipophilic pH indicators were made by covalently binding the xanthene dyes fluorescein, eosin or dichlorofluorescein to the amino group of phosphatidylethanolamine. The probes were incorporated into phospholipid vesicles and the effect of pH on the fluorescence was characterized. Fluorescence was measured at a single emission wavelength during excitation at two wavelengths, and the ratio of the intensities was calculated. The experimentally observed $pK_{obs.}$ values were determined by fitting the fluorescence ratios to the Henderson–Hasselbalch equation. All three probes acted as pH indicators, and the eosinyl-, dichlorofluoresceinyl- and fluoresceinyl-phosphatidylethanolamines had $pK_{obs.}$ values of 3.5, 6.3 and 7.5 respectively. At physiological salt concentrations, changes in the composition of the vesicle membrane had little effect on these values. We concluded that these probes were promising candidates for the measurement of pH values at cell surfaces.

INTRODUCTION

The control of cytoplasmic pH is important for cellular function and development [1]. In order to maintain this internal equilibrium while normal metabolism is producing acid, there must be a continuous net efflux of protons across the plasma membrane [2]. This suggests that the external pH adjacent to the membrane of a living cell may be substantially lower than that further from the cell [3]. The lowest pH values will be found at the surface in an interfacial region which can be considered as the environment of the bound proteins of the plasma membrane. It is this interfacial pH that will be measured by membrane-bound probes such as those described in the present paper. There will also be a pericellular region over which the pH increases to that of the bulk solution. The depth of this zone will vary with cellular metabolic activity and environment, but it is unlikely to extend more than about 1 μm from the cell surface [3]. As the action of certain catabolic enzymes would be favoured by an acidic microenvironment [4], the existence of such local regions of low pH would have important implications when considering possible mechanisms of turnover of the extracellular matrix.

The measurement of pH values at cell surfaces using microelectrodes presents technical difficulties, owing to the size of the probe and the need for precise positioning, but the method has shown that acidic regions occur beneath activated macrophages adsorbed to a collagen substrate and under osteoclasts mediating bone resorption [5,6]. However, an alternative method is needed to probe less-restricted environments and to provide data at higher spatial resolution. Fluorescent pH probes are powerful tools in the study of intracellular pH [7,8], and fluorescent lipids may be suitable for the measurement of pH values at the surface of living cells [9].

We have examined the pH-indicator properties of some xanthene dyes bound to phospholipid vesicles through conjugates to PE. The effects of pH have been characterized by using the fluorescence-ratio method commonly used in microscopic measurements of intracellular pH [7,8]. Although this method offers a number of significant advantages, it appears not to have been used with fluorescein conjugates in model membranes [10,11]. We report here the results of fluorescence-ratio titrations of three xanthene-dye-labelled PEs in phospholipid vesicles. These conjugates have pK values between 3.5 and 7.5, and they are promising probes of cell-surface pH.

MATERIALS AND METHODS

Materials

Sodium fluorescein, fluorescein 5-isothiocyanate, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine and di-isopropylcarbodi-imide were from Fluka. Eosin 5-isothiocyanate and 5- (and 6-) carboxy-2′,7′-dichlorofluorescein were from Molecular Probes. Dioleoylphosphatidylcholine (DOPC) and egg-yolk PE were from Sigma. Phosphatidic acid (PA), from egg lecithin, and phosphatidylserine (PS), from bovine spinal cord, were from Lipid Products. Other reagents were of the purest grades commercially available. Silica-gel plates

Abbreviations used: FLPE, *N*-fluoresceinylphosphatidylethanolamine; EOPE, *N*-eosinylphosphatidylethanolamine; DCPE, 2′,7′-dichlorofluoresceinylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; PE, phosphatidylethanolamine, PA, phosphatidic acid, PS, phosphatidylserine; p.l.c., preparative layer chromatography.

(Merck 5717) for preparative layer chromatography (p.l.c.) were from BDH. T.l.c. sheets (13181) were from Kodak.

Buffers

These were as follows: buffer A, 10 mM-Tris/HCl, pH 7.3; buffer B, 20 mM-Tris/maleate adjusted in the pH range 5–10 with 1 M-HCl; Buffer C, 0.05 M-citric acid adjusted in the pH range 2–7 with 1 M-NaOH. pH values were measured at 37 °C with a Radiometer pH-meter PHM83 with a combined electrode and temperature probe. Buffers contained 0.02% NaN_3 and 0.1 M-NaCl, except in experiments where the ionic strength was varied. Buffers for fluorescence experiments were passed through 0.45 μm-pore-size filters to remove scattering contaminants.

Fluoresceinyl- and eosinyl-phosphatidylethanolamine (FLPE and EOPE)

These were made as described [12,13] by labelling the polar head group of egg-yolk PE with the amino-reactive dyes, fluorescein isothiocyanate and eosin isothiocyanate. The fluorescent lipids were isolated by repetitive p.l.c. until one spot was obtained on t.l.c. The products were stored in chloroform/methanol (1:1, v/v) at −20 °C and re-purified by p.l.c. as necessary. Concentrations of the PE derivatives were measured by phosphorus assay [14].

2′,7′-Dichlorofluoresceinylphosphatidylethanolamine (DCPE)

This was made by allowing PE to react with an activated ester of the fluorophore. 5- (and 6-) Carboxy-2′,7′-dichlorofluorescein (10.2 mg, 20 μmol) and 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-triazine (6.5 mg, 40 μmol) were dissolved in dry dimethylformamide (0.6 ml). Di-isopropylcarbodi-imide (3.2 μl, 20 μmol) was added and the mixture was stirred at 21 °C for 3 h. PE (7.5 mg, 10 μmol) and di-isopropylethylamine (20 μl, 116 μmol) in chloroform (0.75 ml) were added and stirring was continued for 2 h, by which time PE was not detectable on t.l.c. Chloroform (5 ml) was added, and the precipitated di-isopropylurea was removed by filtration. The organic phase was extracted twice with 5% (w/v) citric acid (10 ml) and twice with water (10 ml) before drying over anhydrous $MgSO_4$. DCPE was purified by p.l.c. in chloroform/methanol/acetic acid (70:30:3, by vol.). On t.l.c. there was one fluorescent phospholipid spot, but a trace of free dye was also visible.

Phospholipid vesicles

These were made in buffer A by the cholate method [15]. In most experiments the vesicles contained 0.2 mol% of fluorescent PE and up to 15 mol% of charged phospholipid, the remaining lipid being DOPC. Some experiments were made with vesicles in which part of the PC was replaced by cholesterol. Vesicle preparations were passed through 0.22 μm-pore-size filters and used within 24 h.

Fluorescence measurements

These were made at 37 °C in a Perkin–Elmer LS-3 spectrofluorimeter equipped with Rank Brothers electronic stirrers. Buffer B or buffer C (3 ml) at the chosen pH was allowed to warm to 37 °C, and blank values were recorded before adding the fluorescent vesicles. The final concentration of fluorophore was 0.1–0.5 μm. Fluorescence was recorded at a fixed emission wavelength with two excitation wavelengths [16]. Fluorescein emission was at 526 nm and excitation was at 450 and 495 nm. Eosin emission was at 560 nm and excitation was at 470 and 532 nm. Dichlorofluorescein emission was at 540 nm and excitation was at 450 and 520 nm. The fluorescence ratio is defined as the fluorescence at the longer excitation wavelength divided by that at the shorter wavelength.

Calculation of $pK_{obs.}$

The theoretical basis of fluorescence-ratio titrations is analogous to that given by Grynkiewicz *et al.* [17] for calcium indicators. Briefly, it is assumed that the fluorescence spectrum changes with pH because of the ionization of a single group. In the case of fluorescein at pH values above pH 5.5, this is the hydroxy group on the xanthene ring [18]. If the dye concentration is sufficiently low, the fluorescence, F, observed at wavelengths λ_1 and λ_2 will be proportional to the concentrations of the emitting species, and we can write:

$$F_1 = S_{a1} \cdot C_a + S_{b1} \cdot C_b \quad (1a)$$

$$F_2 = S_{a2} \cdot C_a + S_{b2} \cdot C_b \quad (1b)$$

where C_a and C_b are the concentrations of the acidic and basic forms of the dye respectively, and the proportionality coefficients are S_{a1} for the acidic dye at λ_1, S_{a2} for the acidic dye at λ_2, S_{b1} for the basic dye at λ_1 and S_{b2} for the basic dye at λ_2. However, C_a and C_b are related by the equation for acid dissociation:

$$C_a = C_b \cdot [H^+]/K_{app.} \quad (2)$$

where $[H^+]$ is the hydrogen-ion concentration and $K_{app.}$ is the apparent dissociation constant. Thus, if the fluorescence ratio, R, is defined as F_1/F_2, and eqn. (2) is substituted into eqns. (1a) and (1b) we obtain:

$$R = (S_{a1} \cdot [H^+]/K_{app.} + S_{b1})/(S_{a2} \cdot [H^+]/K_{app.} + S_{b2}) \quad (3)$$

Eqn. (3) can be rearranged to:

$$[H^+]/K_{app.} = (S_{b2}/S_{a2}) \cdot [R - S_{b1}/S_{b2}]/[S_{a1}/S_{a2} - R] \quad (4)$$

S_{a1}/S_{a2} is simply the limiting value that R can have in acidic solutions and so may be written as R_a, whereas S_{b1}/S_{b2} is the analogous limiting ratio R_b in basic solutions. Eqn. (4) can be written in the form:

$$R = (R_a \cdot [H^+] + R_b \cdot K_{obs.})/([H^+] + K_{obs.}) \quad (5)$$

where $K_{obs.}$ is the experimental dissociation constant. The fluorescence ratio (R) values at each pH were fitted to eqn. (5) by non-linear regression [19]. The experimental and apparent pK values are related as follows:

$$pK_{app.} = pK_{obs.} + \log(F_b/F_a)$$

where F_b and F_a are the limiting fluorescence values for the basic and acidic forms of the probe respectively at λ_2, in this case the shorter wavelength.

RESULTS

FLPE

The fluorescence excitation spectrum of FLPE in DOPC vesicles changed with pH in a manner very similar to the absorbance changes reported for FLPE in asolectin vesicles [10]. When the fluorescence ratios were plotted versus pH (Fig. 1), the Henderson–Hasselbalch equation

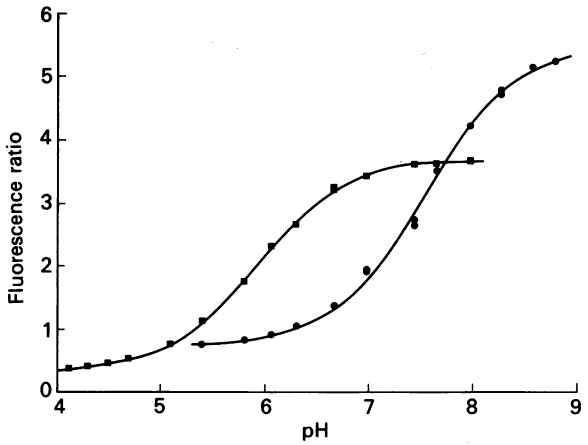

Fig. 1. pH-dependence of the fluorescence ratio of sodium fluorescein and of FLPE in phospholipid vesicles

The fluorescence of sodium fluorescein (■) and of FLPE (●) in DOPC vesicles was measured at 526 nm during excitation at 450 nm and at 495 nm. The lines were calculated by fitting the values of the fluorescence ratio, F_{495}/F_{450}, to the Henderson–Hasselbalch equation, as described in the text.

was closely obeyed, and the fluorescein moiety had $pK_{obs.} = 7.5$. Compared with the water-soluble sodium fluorescein, which had $pK_{obs.} = 5.9$, the titration curve was displaced towards more alkaline pH values.

In an uncharged membrane of DOPC such an increase in pK_a would occur if the probe were located in a region of decreased dielectric constant [20]. However, fluorescent indicators also exhibit pK_a shifts when bound to electrically charged membranes [20,21] and macromolecules [22]. We examined, therefore, the effects of membrane composition and charge on $pK_{obs.}$. Vesicles were made with up to 15 mol% of PE, PA or PS and with 40 mol% of cholesterol. In the case of PE, the vesicles remained neutral, but vesicles with 15 mol% of PA and PS probably had surface potentials close to −30 mV in 0.1 M-NaCl [23]. Despite these changes, the $pK_{obs.}$ values of FLPE remained close to 7.5 (Table 1). Similarly, incorporation of 40 mol% of cholesterol had only slight effects on $pK_{obs.}$ (Table 1). At low ionic strengths, vesicles with 15 mol% of PA or PS showed an increase in $pK_{obs.}$ of 0.4 unit (Table 2). At 37 °C this shift in pK is equivalent to a change in membrane potential of −25 mV. As expected, vesicles made with 15 mol% of PE were unaffected by changes in NaCl concentration (Table 2).

These results suggested that the indicator properties of FLPE were relatively insensitive to lipid composition within the range encountered in biological membranes [24]. However, the value of $pK_{obs.}$ limits the usefulness of FLPE as a probe of cell-surface pH. Fig. 1 shows that FLPE is most responsive at pH values above 6.5, whereas studies with microelectrodes have shown that the pH can fall below pH 4 in a restricted environment adjacent to living cells [6].

EOPE

The pK_a values of halogenated xanthene dyes are substantially lower than that of fluorescein. Thus, 5-carboxyfluorescein has $pK_a = 6.5$, whereas the brominated derivative 5-carboxyeosin has pK_a in the range 4–5 [25]. If, like fluorescein, conjugation to PE and incorporation into vesicles leads to an increase in pK_a, the pH response of EOPE would be in the range 5–7. However, when EOPE was titrated using the fluorescence-ratio method (Fig. 2), the probe did not behave as expected and had a very low $pK_{obs.}$ value of 3.5. Although EOPE may be applicable to the measurement of pH values under macrophages and osteoclasts [6], it has too low a value of $pK_{obs.}$ to allow measurements above pH 5.

DCPE

It seemed likely that the anomalous behaviour of EOPE was due in some way to a shielding effect of the

Table 1. Mean values of $pK_{obs.}$ of sodium fluorescein and of FLPE in phospholipid vesicles

Sodium fluorescein or phospholipid vesicles containing 0.2 mol% of FLPE and up to 15 mol% of PE, PA or PS were diluted into buffer B to give 0.1–0.5 μM-fluorophore. Fluorescence was measured at 526 nm during excitation at 495 nm and at 450 nm. Values of R, the fluorescence ratio F_{495}/F_{450}, were fitted to the Henderson–Hasselbalch equation by non-linear regression. Two or three independent experiments were performed. The values of $pK_{obs.}$ were always within 0.1 of the mean.

Fluorescein preparation	Mean $pK_{obs.}$
Sodium fluorescein	5.9
FLPE/DOPC	7.5
FLPE/DOPC/5% PE	7.5
FLPE/DOPC/10% PE	7.4
FLPE/DOPC/15% PE	7.4
FLPE/DOPC/5% PA	7.6
FLPE/DOPC/10% PA	7.7
FLPE/DOPC/15% PA	7.7
FLPE/DOPC/5% PS	7.6
FLPE/DOPC/10% PS	7.6
FLPE/DOPC/15% PS	7.6
FLPE/DOPC/40% cholesterol	7.3
FLPE/DOPC/15% PA/40% cholesterol	7.7

Table 2. Effect of salt concentration on $pK_{obs.}$ of FLPE in phospholipid vesicles

Fluorescence-ratio titrations were done as in Table 1. The buffer was 20 mM-Tris/maleate containing 20 mM-, 100 mM- or 200 mM-NaCl. One titration was made at each NaCl concentration.

Vesicle	[NaCl] (mM)	$pK_{obs.}$
FLPE/DOPC/15% PE	20	7.3
	100	7.4
	200	7.3
FLPE/DOPC/15% PA	20	7.8
	100	7.6
	200	7.4
FLPE/DOPC/15% PS	20	7.8
	100	7.5
	200	7.4

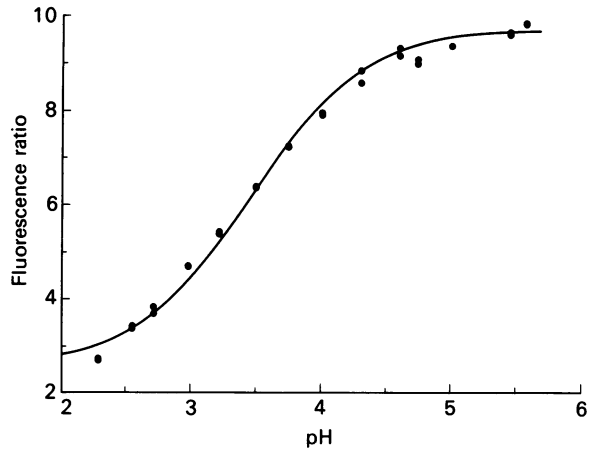

Fig. 2. pH-dependence of the fluorescence ratio of EOPE in phospholipid vesicles

The fluorescence of EOPE in DOPC vesicles was measured at 560 nm during excitation at 470 nm and at 532 nm. The line was calculated by fitting the values of the fluorescence ratio, F_{532}/F_{470}, to the Henderson–Hasselbalch equation.

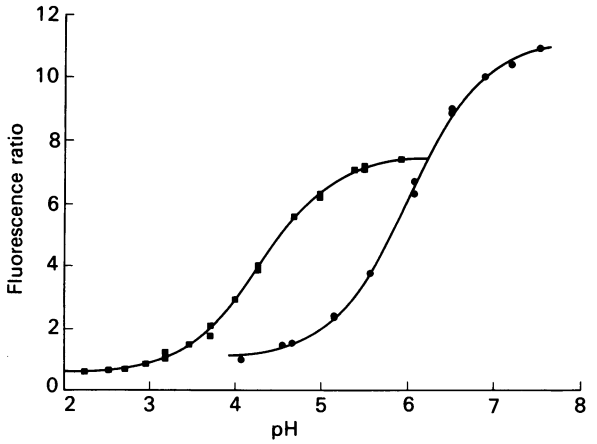

Fig. 3. pH-dependence of the fluorescence ratio of 5- (and 6-) carboxy-2′,7′-dichlorofluorescein and of DCPE in phospholipid vesicles

The fluorescence of 5- (and 6-) carboxy-2′,7′-dichlorofluorescein (■) and of DCPE (●) in DOPC vesicles was measured at 540 nm during excitation at 450 nm and at 520 nm. The lines were calculated by fitting the values of the fluorescence ratio, F_{520}/F_{450}, to the Henderson–Hasselbalch equation.

bromine atoms on either side of the ionizing hydroxy group. We considered 2′,7′-dichlorofluorescein to be a structural compromise between eosin and fluorescein: the two chlorine atoms will reduce the pK_a, but they should allow much greater exposure of the hydroxy group. An amine-reactive derivative of 2′,7′-dichlorofluorescein was not commercially available, but we were able to couple the dye to PE with an activated ester. In fluorescence-ratio titrations the free dye had a $pK_{obs.}$ of 4.3, but on coupling to PE and incorporation into phospholipid vesicles the value rose to 6.0 (Fig. 3). As observed with FLPE, the $pK_{obs.}$ of DCPE was insensitive to the overall charge of the membrane (Table 3).

Table 3. $pK_{obs.}$ of dichlorofluorescein carboxylate and of DCPE in phospholipid vesicles

Experiments were performed as described in Table 1, except that fluorescence was measured at 540 nm during excitation at 520 and at 450 nm. Two or three independent experiments were made. Individual values of $pK_{obs.}$ were within 0.05 of the mean.

Dichlorofluorescein preparation	Mean $pK_{obs.}$
Dichlorofluorescein carboxylate	4.3
DCPE/DOPC	6.3
DCPE/DOPC/15% PE	6.3
DCPE/DOPC/15% PA	6.3
DCPE/DOPC/15% PS	6.5

DISCUSSION

The aim of the present study was to develop fluorescent probes to measure the pH values influencing local enzyme activity in the interfacial and pericellular regions around living chondrocytes [9]. These cells can be stimulated to resorb their extracellular matrix by agents such as retinol [26], bacterial lipopolysaccharides [27] and the cytokines interleukin 1 [28] and tumour necrosis factor [29]. Characterization of proteoglycan fragments released from cartilage stimulated by interleukin 1 suggests that proteolytic enzymes are involved in the breakdown [30], but these proteinases have not been identified. There is some evidence that the proteolytic activity in stimulated cartilage is not freely diffusable [31], but this could reflect the denaturation of the enzyme(s) on diffusion away from a local acidic environment around the cell. Such behaviour might be expected of the lysosomal enzymes cathepsins B [32], D [33] and L [34], although there is no evidence to support their involvement in cartilage resorption [35].

As probes of pH values at the surfaces of living cells, xanthene-dye-labelled PEs have a number of attractive features. They are highly fluorescent at non-lethal wavelengths and they can be readily and stably incorporated into biological membranes [11]. In addition, fluorescein and its analogues have been used extensively in fluorescence-ratio imaging microscopy [7,8]. This technique not only has excellent spatial resolution, but also has the major advantage that the ratio relationship normalizes for optical pathlength, local probe concentration, illumination intensity and photobleaching [8].

The choice of suitable pH probes had to take account of the alkaline shifts in pK_a which occurred on incorporation into lipid vesicles. Similar shifts have been observed in other fluorescent indicator–vesicle systems [21,36,37]. The value of $pK_{obs.}$ for FLPE is similar to that reported [10] for the probe in asolectin vesicles. However, our value was determined by fluorescence-ratio titration and when corrected gave a $pK_{app.}$ of 8.1. We cannot account for this discrepancy, but it has been a consistent observation. Sources of error in the determination of $pK_{obs.}$ include the possibility that the inner compartment of the vesicles had not equilibrated with the outer buffer. This seems unlikely, as in preliminary experiments the addition of the ionophore carbonyl cyanide *m*-chlorophenylhydrazone did not alter the value of $pK_{obs.}$.

We were also concerned that the probes acted as true

pH indicators and were not sensitive to changes in local surface charge. If this were so, regions of higher charge density would appear to have a different pH. However, when charged lipids were added to the vesicles, the effects on $pK_{obs.}$ were small (Tables 1 and 3) and at physiological salt concentrations the indicator properties of the probes will be insensitive to changes in membrane potential.

These studies suggest that the xanthene-dye-labelled phosphatidylethanolamines are promising probes for fluorescence-ratio measurements of interfacial pH in cellular systems. Fluoresceinyl-PE is suitable for measurements at physiological pH and DCPE responds from pH 7 down to pH 5. At more acidic pH values, eosinyl-PE will allow measurements down to pH 3. Thus these three probes are sensitive to pH over the whole range of values likely to be encountered under physiological conditions.

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